ELSEVIER

Comparative Biochemistry and Physiology Part A 121 (1998) 209–214

CBP

In vivo prevention of cyclophosphamide-induced Ca^{2+} dependent damage of rat heart and liver mitochondria by cyclosporin A

Ibrahim A. Al-Nasser *

Department of Biochemistry, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia

Received 5 January 1998; received in revised form 14 May 1998; accepted 1 June 1998

Abstract

The use of Cyclophosphamide, an anti-cancer and immunosuppressant drug, is accompanied by a number of side effects. Rats injected with a single dose of cyclophosphamide (200 mg kg^{-1} body weight) showed an increase in the levels of serum glutamate-oxaloacetate transaminase, serum glutamate-pyruvate transaminase, glucose-6-phosphate dehydrogenase and creatine phosphokinase isoenzyme by 53, 24, 55 and 135%, respectively. Also the ability of heart or liver mitochondria to retain accumulated Ca^{2+} and tetraphenylphosphonium ion was sharply affected in treated rats. Rats injected with the same dose of cyclophosphamide plus cyclosporin A (500 μg kg^{-1} body weight) showed reduction in the levels of those enzymes by about 44, 21, 43 and 57%, respectively compared to cyclophosphamide-treated rats. Cyclosporin A treatment also restored mitochondrial ability to retain accumulated Ca^{2+} and tetraphenyl phosphonium ions nearly to the level of untreated rats. We suggest that cyclophosphamide induced cardio and hepatotoxicity by increasing heart and liver inner mitochondrial membrane permeability to Ca^{2+}. The protective effect of cyclosporin A against cyclophosphamide-induced damage also support this suggestion.

Keywords: Cyclophosphamide; Heart; Liver; Mitochondria; Cyclosporin A; Calcium; Pore; Membrane potential

1. Introduction

In heart and liver mitochondria the simultaneous operation of the Ca^{2+} uniporter, responsible for Ca^{2+} accumulation, and the efflux mechanism, which involves distinct Na^+-independent and Na^+-dependent process for Ca^{2+} release, led to the concept that a continuous cycling of Ca^{2+} occurs across the innermitochondrial membrane [9,26,29]. Several enzymes of oxidative metabolism are sensitive to Ca^{2+}. Three matrix enzymes from liver mitochondria; pyruvate dehydrogenase, isocitrate dehydrogenase and α ketoglutarate dehydrogenase are activated by free Ca^{2+} in excess of 0.1–10 μM [25,26].

High levels of cytosolic Ca^{2+} opens a non-specific pore in the inner mitochondrial membrane. When opens, the pore renders mitochondria leaky leading to uncoupling of energy transduction and the release of matrix free Ca^{2+} [4,7,10,16,30]. Under pathological conditions associated with high cellular Ca^{2+}, the impairment of mitochondrial ATP production by pore opening may be an important contributory factor in Ca^{2+} induced cell death [31].

Cyclophosphamide is an alkylating agent that is in use as anti-cancer and immunosuppressant drug [27]. Cyclophosphamide metabolism is responsible for its immunosuppressing effect [27]; the drug itself lacks immunosuppressant effect and must first be activated by cytochrome P_{450} in the liver [35]. The use of cyclophosphamide is accompanied by a number of side effects such as nausea, vomiting, alopecia, leukopenia and cardiac necrosis [12]. The toxicity and pharmacology of cyclophosphamide have been reviewed by Gershwin et al. [14] and Bagley and Bostick [6].

* Tel.: +966 1 4675796; fax: +966 1 4675791.

1095-6433/98/$ - see front matter
PII S1095-6433(98)10135-6

Exhibit A

Cyclosporin A (CsA) is a well-known immunosupressive drug [13,21]. The effect of CsA on Ca^{2+} retention by liver, heart and kidney mitochondria, has been the subject of many studies. Addition of CsA to respiring mitochondria of liver, heart or kidney under conditions of Ca^{2+} overload induced maintenance of membrane potential, inhibited matrix volume expansion, and increased mitochondrial ability to retain pre-accumulated Ca^{2+} [3,11,18,33]. Nazareth et al. [28] suggested that CsA is a potent inhibitor of the non specific pore in inner mitochondrial membrane.

This investigation examined the effect of cyclophosphamide administration on liver and heart mitochondria, in vivo, and the impact of CsA presence.

2. Materials and methods

A total of 36 adults male Sprague–Dawly rats from our departmental facility were divided into three equal groups. One group received i.p. injection of cyclophosphamide (200 mg kg^{-1} body weight), second group received the same dose of cyclosphamide plus CsA (500 $\mu g\ kg^{-1}$ body weight) and the third group (control) injected with an equivalent volume of normal saline. The experiment was repeated with a total of 48 adults rats divided into the same three groups as above plus fourth group injected with the same dose of CsA alone. All rats were housed in standard conditions and allowed unlimited access to food and water.

On day 7, rats were anesthetized with ether and blood was collected from tails for serum glutamate-oxaloacetate transaminase (SGOT), serum glutamate-pyruvate transaminase (SGPT), glucose-6-phosphate dehydrogenase (G6P-DH) and creatine phosphokinase isoenzyme (CPK_2-MB) estimation. Rats were then killed by cervical fracture, and their livers, hearts were immediately excised for isolation of mitochondria as described in detail elsewhere [1]. The final mitochondrial pellets were resuspended in MST buffer (210 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl, pH 7.2). Protein was measured by a modified biuret method [24].

Ca^{2+} efflux was measured by the Ca^{2+} indicator arsenazo III with a PYE-Unicam spectrophotometer set at 675–685 nm. Mitochondria (1 mg ml^{-1}) was pre-incubated at 25°C in standard medium containing: 120 mM KCl–10 mM Tris–Hepes (pH 7.2), 60 μM arsenazo III, rotenone (1 $\mu g\ mg^{-1}$ protein), phosphate (P_i) (either 1 or 2 mM for liver or heart mitochondria, respectively). Ca^{2+} was then added to a total load of either 20 or 50 nmol mg^{-1} protein for liver or heart mitochondria, respectively. The uptake of Ca^{2+} was initiated by addition of 4 mM succinate.

Tetraphenyl phosphonium ion (TPP^+) uptake, estimated with a TPP^+-sensitive electrode prepared in our laboratory as described by Kamo et al. [22], was used to monitor changes in isolated liver and heart mitochondria membrane potential. Accumulation and release of TPP^+ by coupled mitochondria is directly related to membrane potential [22]. Mitochondria (1 mg ml^{-1}) was incubated in medium (3 ml) containing, 120 mM KCl, 10 mM Tris–Hepes (pH 7.2), rotenone (1 $\mu g\ mg^{-1}$ protein), 3 μM TPP^+, P_i 1 or 2 mM for liver or heart mitochondria, respectively, and 20 or 50 nmol Ca^{2+} mg^{-1} protein for liver or heart mitochondria, respectively. TPP^+ influx was started by addition of 4 mM succinate.

SGOT, SGPT levels were measured according to the method of Retiman and Frankel [32], G6P-DH level was measured according to the method of Kornberg and Horecker [23] CPK_2-MB level was measured according to the method of Kachmar and Moss [20].

Rotenone and TPP^+ were purchased from Aldrich (Gillingham, UK). Arsenazo III and cyclophosphamide were obtained from Fluka AG. (Buchs, Switzerland). Cyclosporin A was a kind gift from Dr A. Al-Nasser of the King Faisal Specialist Hospital, Riyadh. All other chemicals were purchased from the Sigma (St. Louis, MO).

3. Results

Fig. 1 shows the ability of heart mitochondria to accumulate and retain Ca^{2+}. Mitochondria isolated from cyclophosphamide-treated rats accumulated only about 25 nmol Ca^{2+} mg^{-1} protein in the first minute followed by a gradual release of Ca^{2+} (Fig. 1, upper trace). In contrast, mitochondria isolated from control rats (or CsA treated rats) accumulated and retained all Ca^{2+} during the entire experiment (Fig. 1, bottom trace). Similar ability to accumulate and retain Ca^{2+} was observed with mitochondria isolated from cyclophosphamide plus-CsA treated rats (Fig. 1, dotted trace). The ability of heart mitochondria to accumulate and retain TPP^+ was also examined (Fig. 2). Mitochondria from cyclophosphamide treated rats accumulated TPP^+ initially but was unable to retain it (Fig. 2, upper trace). By contrast, mitochondrial of normal, (or CsA treated rats) or cyclophosphamide plus CsA treated rats (Fig. 2, bottom trace or dotted trace, respectively) were able to accumulate and retain TPP^+ for the duration of the experiment.

Mitochondria of the liver showed similar pattern of damage/protection by the injection of cyclophosphamide/cyclophosphamide-plus-CsA (Figs. 3 and 4). Liver mitochondria isolated from cyclophosphamide-injected rats accumulated about 15 nmol Ca^{2+} mg^{-1} protein, then slowly released it (Fig. 3, top trace). Mitochondria of either control (or CsA treated rats)

(Fig. 3, bottom trace) or cyclophosphamide-plus-CsA-treated rats (Fig. 3, dotted trace) accumulated and retained Ca^{2+}. Fig. 4 shows the ability of those livers mitochondria to accumulated and retain TPP^+. Liver mitochondria from cyclophosphamide-treated rats accumulated TPP^+ in the first minute then slowly released it (top trace). Whereas mitochondria from either control (or CsA treated rats) (bottom trace) or cyclophosphamide plus CsA treated rats (dotted trace) were able to accumulate and retain TPP^+.

The effect of cyclophosphamide treatment, ± CsA on the levels of enzymes (GOT), (GPT), (G6P-DH) and (CPK_2-MB) were tested. The levels of those enzymes estimated from blood samples collected in day 7 post injection and compared to control (Table 1). The serum levels of (GOT), (GPT), (G6P-DH) and (CPK_2-MB) increased by 53, 24, 55 and 135%, respectively, in cyclophosphamide-treated rats. In contrast, levels of those enzymes in CsA treated rats or cyclophosphamide-plus-CsA-treated rats were lower than those of control rats.

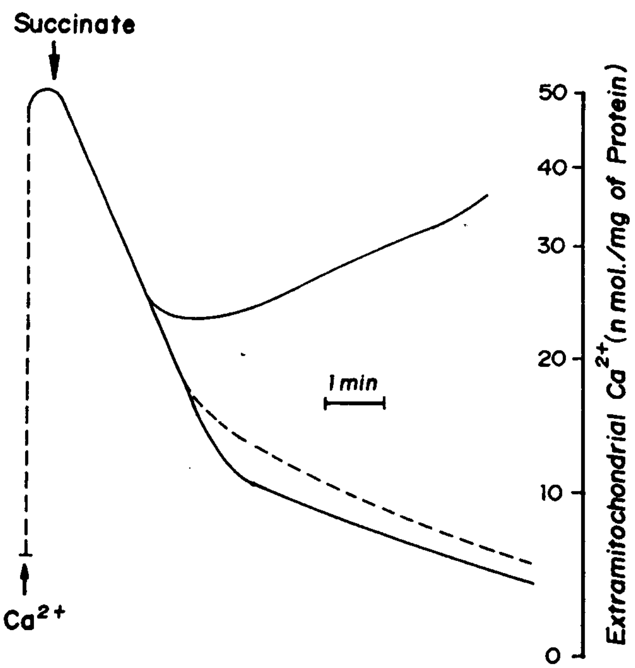

Fig. 1. Effect of cyclophosphamide on the capacity of heart mitochondria to accumulate and retain Ca^{2+}. Heart mitochondria (1 mg ml^{-1}) isolated from either cyclophosphamide treated rats (top trace) or cyclophosamide plus CsA treated rats (dotted trace) or from normal rats (or CsA treated rats) (bottom trace) were incubated in assay buffer (3 ml final volume) containing, 120 mM KCL–10 mM Tris–Hepes (pH 7.2), 1 μg rotenone mg^{-1} protein, 60 μM arsenazo III, 2 mM phosphate, 50 nmol Ca^{2+} mg^{-1} protein. The uptake of Ca^{2+} was initiated by the addition of 4 mM succinate as indicated. These results are from two studies and are representatives of four experiments for each study.

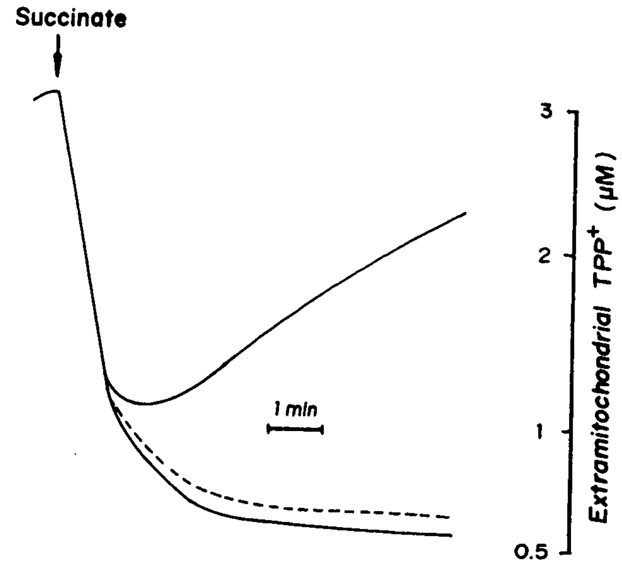

Fig. 2. The ability of heart mitochondria to accumulate and retain TPP^+. Heart mitochondria (1 mg ml^{-1}) isolated from either cyclophosphamide treated rats (top trace) or cyclophosphamide plus CsA treated rats (dotted trace) or from normal rats (or CsA treated rats) (bottom trace) were incubated in assay buffer (3 ml final volume) containing, 120 mM KCL–10 mM Tris–Hepes (pH 7.2), 2 mM phosphate, 50 nmol Ca^{2+} mg^{-1} protein, 1 μg rotenone mg^{-1} protein, 3 μM TPP^+. The uptake of TPP^+ was initiated by the addition of 4 mM succinate as indicated. These results are from two studies and are representatives of six experiments for each study.

4. Discussion

Cyclophosphamide is an anti-cancer and immunosuppressant drug that is known to have a number of side effects. Many studies reported that treatment with cyclophosphamide produced severe liver damage but the mechanism has been difficult to establish [5,15,17,19].

Previously we reported that some anti-cancer drugs (cisplatin-adriamycin), which caused nephrotoxicity and cardiotoxicity, were involved in opening of pore in the inner mitochondrial membrane of kidney and heart [1,8]. In this study, we report the possible involvement of cyclophosphamide treatment in opening the same mitochondrial pore in heart and liver. Rats injected with a single dose of cyclophosphamide (200 mg kg^{-1} body weight) showed increase in the levels of serum (GOT), (GPT), (G6P-DH) and (CPK_2-MB) by 53, 24, 55 and 135%, respectively (Table 1). This is most likely the results of toxic side effect of cyclophosphamide administration as elevated levels of these enzyme is associated with certain types of heart damage such as myocardial infraction, myocarditis, and heart failure [20,34].

Adminstration of cyclophosphamide to rats sharply affected the heart, and to lesser extent the liver mitochondria ability to retain accumulated Ca^{2+} (Figs. 1 and 3) or TPP (Figs. 2 and 4). We postulated in

previous studies that a pore in inner mitochondrial membrane of liver, heart and kidney is ascillating between close and open state [2,15,10]. High intramitochondrial free Ca^{2+} stabilizes the open state of those pores leading to increased permeability and consequent uncoupling of mitochondrial linked ATP synthesis. Ca^{2+} overload is the pathogenetic mechanism in certain types of cell injury. It has often been suggested that high Ca^{2+} may be detrimental to mitochondrial energy transduction in those forms of injury associated with excess cellular Ca^{2+} and that mitochondrial dysfunction might contribute to the progression of the injury [28,31]. In this kind of disease increasing the cytosolic Ca^{2+} will lead to increase of intramitochondrial Ca^{2+} and dysfunction mitochondria possibly by opening the pore.

There is increasing evidence suggesting that pore inhibitory agents, such as CsA, could protect heart, liver, and kidney mitochondria from functional alterations associated with matrix Ca^{2+} overload [3,11,18]. Inclusion of CsA appeared to provide protection against toxic effects of cyclophosphamides as examined by both levels of metabolic enzymes or mitochondrial properties. In rats treated with cyclophosphamide plus CsA, levels of serum (GOT), (GPT), (G6P-DH) and (CPK_2-MB) were reduced by about 44, 21, 43 and 57%, respectively (compared to cyclophosphamide treated rats). In rats treated with CsA alone the level of those enzymes reduced by about 16, 4, 8 and 9%, respectively (compared to control). The ability of liver and heart mitochondria isolated from cyclophosphamide plus CsA treated rats to retain accumulated Ca^{2+} and TPP^+ was also restored to almost the level of control (Figs. 1–4, dotted traces). The apparent protective effect of CsA against cyclophosphamide induced damage in heart and liver mitochondria and the increased tolerance of their mitochondria to cyclophosphamide, indicated a likely relationship between cyclophosphamide toxicity and permeability of inner mitochondrial membrane of liver and heart.

In conclusion, the present study showed that biochemical and haematological parameters could be used as markers for cyclophosphamide induced cardio-and hepatotoxicity. Cyclophosphamide toxicity might be the result of increased permeability of heart and liver inner mitochondrial membrane. This possibility is supported by the protective effect of CsA against cyclophosphamide induced damage to mitochondria.

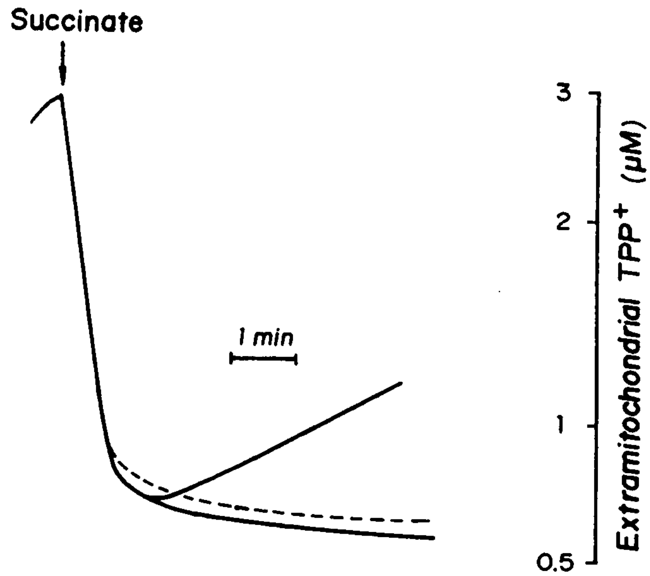

Fig. 4. The ability of liver mitochondria to accumulated and retain TPP^+. Liver mitochondria (1 mg ml^{-1}) isolated from either cyclophosphamide treated rats (top trace) or cyclophosphamide plus CsA treated rats (dotted trace) or from normal rats (or CsA treated rats) (bottom trace) were incubated in assay buffer (3 ml final volume) containing, 120 mM KCl–10 mM Tris–Hepes (pH 7.2), 1 mM phosphate, 20 nmol Ca^{2+} mg^{-1} protein, 1 μg rotenone mg^{-1} protein, 3 μM TPP^+. The uptake of TPP^+ was initiated by the addition of 4 mM succinate as indicated. These results are from two studies and are representatives of eight experiments for each study.

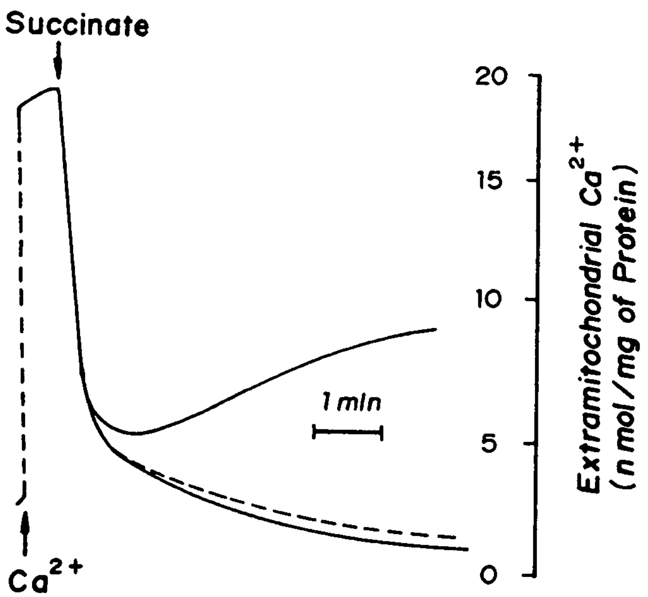

Fig. 3. Effect of cyclophosphamide on the capacity of liver mitochondria to accumulate and retain Ca^{2+}. Liver mitochondria (1 mg ml^{-1}) isolated from either cyclophosphamide treated rats (top trace) or cyclophosphamide plus CsA treated rats (dotted trace) or from normal rats (or CsA treated rats) (bottom trace) were incubated in assay buffer (3 ml final volume) containing, 120 mM KCl–10 mM Tris–Hepes (pH 7.2), 1 μg rotenone mg^{-1} protein, 60 μM arsenazo III, 1 mM phosphate, 20 nmol Ca^{2+} mg^{-1} protein. The uptake of Ca^{2+} was initiated by the addition of 4 mM succinate as indicated. These results are from two studies and representatives of four experiments for each study.

Table 1
Cyclophosphamide ($\pm$ CsA)-induced alteration of rats serum enzymes and isoenzyme levels

Group	Serum enzymes and isoenzyme levels, 7 days after treatment (μ l^{-1})			
	GOT	GPT	G6P-DH	CPK_2-MB
Control	73.4 ± 3.2	38.2 ± 1.8	0.84 ± 0.2	21.3 ± 2.5
Cyclophosphamide alone	112.2 ± 5.8*	47.3 ± 2.6*	1.3 ± 0.3*	50.1 ± 3.2*
Cyclophosphamide plus CsA	62.4 ± 2.1**	37.5 ± 2.3**	0.74 ± 0.1**	22.2 ± 4.1**
CsA alone	61.8 ± 1.9	36.8 ± 3.4	0.77 ± 0.2	19.4 ± 3.8

Values present are in mean $\pm$ S.E.M., $n = 12$–24.
* Significantly different from control and CsA alone, $P < 0.05$.
** Significantly different from cyclophosphamide alone, $P < 0.05$.

Acknowledgements

The technical assistance of Abdin A. Wahab is greatly appreciated. The author would like to thank Zahoor A. Javed for typing the manuscript.

References

[1] Al-Nasser I. Prevention of adriamycin aglycone-induced changes of inner mitochondrial membrane. Permeability by cyclosporin A. Med Sci Res 1997;25:249–51.
[2] Al-Nasser I, Campbell P. Phosphate stimulation of Ca^{2+}-induced de polarisation of renal cortex mitochondria. Med Sci Res 1993;21:647–9.
[3] Al-Nasser I, Campbell P. Cyclosporin A inhibition of phosphate-induced calcium release from rat kidney cortex mitochondria. Med Sci Res 1993;21:777–9.
[4] Al-Nasser I, Crompton M. The reversible Ca^{2+}-induced permeabilisation of rat liver mitochondria. Biochem J 1986;239:19–24.
[5] Aubrey D. Massive hepatic necrosis after cyclophosphamide. Br Med J 1970;3:588–93.
[6] Bagley C, Bostick F. Clinical pharmacology of cyclophosphamide. Cancer Res 1973;33:226–35.
[7] Bernardi P, Petronili V. The permeability transition pore as a mitochondrial calcium release channel: a critical appraisal. J Bioenerg Biomembr 1996;24:77–90.
[8] Campbell P, Al-Nasser I. Renal insufficiency induced by cisplatin in rats is ameliorated by cyclosporin A. Toxicology 1996;114:11–7.
[9] Crompton M. Intracellular calcium regulation. Bronnor F, editor. New York: Allan R. Liss, 1990:183–209.
[10] Crompton M, Costi A. Kinetic evidence for a heart mitochondria pore activated by Ca^{2+}, inorganic phosphate and oxidative stress. A potential mechanism for mitochondrial dys function during cellular Ca^{2+} overload. Eur J Biochem 1988;178:489–501.
[11] Cromton M, Ellinger H, Costi A. Inhibition by cyclosporin A of a Ca^{2+}-dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress. Biochem J 1988;255:357–60.
[12] Fisher D, Knobf M, Durivageh H. The cancer chemotherapy hand book. St. Louis. MO, Mosby-year book, 1993.
[13] Fliri H, Baumann G, Enz A, Kallen J, Luyten M, Mikol V, Movva R, Quesniaux V, Screier M, Walkinshaw M, Wenger R, Zeneke G, Zurini M. Cyclosporins structure-activity relationships. Ann NY Acad Sci 1993;696:47–53.
[14] Gershwin M, Goetzl E, Steinberg A. Cyclophosphamide: use in practice. Ann Int Med 1974;80:531–42.
[15] Goldberg J, Lidsky M. Cyclophosphamide-associated hepatotoxicity. South Med J 1985;78:222–3.
[16] Griffith E, Halestrap A. Further evidence that cyclosporin A protects mitochondria from calcium overload by inhibiting a matrix peptidyl-prolyl cis-trans isomerase. Biochem J 1991;274:611–4.
[17] Gustafsson L, Eriksson L, Dahl M, Eleborg L, Ericzon B, Nyberg A. Cyclophosphamide-induced acute liver failure requiring transplantation in a patient with genetically deficient debrisoquine metabolism: a causal relationship. J Int Med 1996;24:311–4.
[18] Halestrap A, David A. Inhibition of Ca^{2+} induced large-amplitude swelling of liver and heart mitochondria by cyclosporin is probably caused by the inhibitor binding to mitochondrial-matrix peptidyl-prolyl cis-trans isomerase and preventing it interacting with the adenine nucleotide translocase. Biochem J 1990;268:153–60.
[19] Honjo I, Suou T, Hirayama C. Hepatotoxicity of cyclophosphamide in man: pharmaco-kinetic analysis. Res Commun Chem Pathol Pharmacol 1988;61:149–65.
[20] Kachmar J, Moss D. Enzymes. In: Teitz W, editor. Fundamentals of Clinical Chemistry. Philadelphia: Saunders, 1982:565–95.
[21] Kahan B. Cyclosporin. New Engl J Med 1989;321:1725–38.
[22] Kamo N, Muratsugo M, Ruyi H, Kobatake J. Membrane potential of mitochondria measured with an electrode sensitive to tetraphenyl phosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state. J Membr Biol 1979;48:105–21.
[23] Kornberg A, Horecker B. Methods in Enzymology, vol. 1. New York: Academic Press, 1955:323.
[24] Koroger A, Klingenberg M. A modified biuret method for the determination of protein. Biochem Z 1966;344:317–38.
[25] McCormack J. Characterisation of the effect of Ca^{2+} on the intramitochondrial Ca^{2+}-sensitive enzymes from rat liver and within rat liver mitochondria. Biochem J 1985;231:581–95.
[26] McCormack J, Halestrap A, Denton R. Role of calcium ions in regulation of mammalian intramitochondrial metabolism. Physiol Rev 1990;70:391–425.
[27] Moore M. Clinical pharmacokinetics of cyclophosphamide. Clin Pharmacokin 1991;20:194–208.
[28] Nazareth W, Yafei M, Crompton M. Inhibition of anoxia-induced injury in heart myocytes by cyclosporin A. J Mol Cell Cardiol 1991;23:1351–4.
[29] Nedegaurd J. Na^{+}-dependent regulation of extramitochondrial Ca^{2+} by rat-liver mitochondria. Eur J Biochem 1984;144:159–68.

[30] Novgordov S, Gudz T. Permeability transition pore of the inner mitochondrial membrane can operate in two open state with different selectivities. J Bioenerg Biomembr 1996;28:139–46.

[31] Peng C, Kane J, Slaub K, Murphy M. Improvement of mitochondrial energy production in ischaemic myocardium by in vivo infusion of ruthenium red. Cardiovasc Pharmac 1980;2:45–54.

[32] Retiman S, Frankel S. A colourimetic method for determination of serum glutamic oxaloacetic and glutamic pyruvic transaminase. Am J Clin Pathol. 1957;28:56–60.

[33] Schweizer M, Schlegel J, Baumgartner D, Richter C. Sensitivity of mitochondrial peptidyl-prolyl *cis–trans* isomerase pyridine nucleotide hydrolysis and Ca^{2+} release to cyclosporin A and related compounds. Biochem Pharmacol 1993;45:641–6.

[34] Singh J, Garge K, Garge D, Chugh K, Lal H. Effect of aspartate and glutamate on experimental myocardial intraction in rats. Indian J Exp Biol 1989;27:621–4.

[35] Sladek N. Metabolism of cyclophosphamide by rat hepatic microsomes. Cancer Res 1971;1:901–8.